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Evaluation of Alternative Antioxidants for Poultry Nutrition: Fractionation of Cashew Seed Shells (*Anacardium occidentale L.*) to Improve Poultry Health, *in Vitro*

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Cashew nut has antioxidant potential with high level of polyphenols, as plant-based antioxidant. This research aims to examine the in vitro fractionation of cashew nut shells as a product that has potential as a source of antioxidants. The treatment consisted of 2 treatments and 3 replications. The treatments used were: P1 (Cashew nut shell fraction with concentrations 80, 160, and 320 ppm), P2 (Vitamin C control concentrations 80, 160, and 320 ppm). The parameters observed in this study were the organoleptic extract, the content of phytochemical compounds in the form of phenol and flavonoid content, antioxidant activity and antioxidant enzyme activity of cashew seed shells, with the resulting output being that the cashew seed shell fraction has a phenol content of 160 mg/g and flavonoids of 300 mg/g, the higher the phenol and flavonoid content. The higher level of activity as an antioxidant, was in accordance with the results of the antioxidant test of the cashew nut shell fraction which has an antioxidant content at a strong level with an IC50 value of 99.459. However, the SOD enzyme content is at a weak level with an IC50 value of 206.250 ppm. The catalase enzyme is at a moderate level with an IC50 value of 121,493 ppm, and in the GPx enzyme content test it is at a weak level with an IC50 value of 279,143 ppm.
Keywords: Cashew nut shells, antioxidants, SOD enzyme, catalase enzyme, GPx enzyme.

INTRODUCTION

Poultry often experiences oxidative stress due to various factors such as an unstable environment, infections, or high production demands (Estévez *et al*., 2015). Oxidative stress occurs when the number of free radicals or reactive oxygen molecules exceeds the body's ability to neutralize them. Free radicals can damage cells and molecules in the livestock's body, leading to cellular damage and physiological disorders. The impact of oxidative stress on poultry involves disruption of the immune system, reduced growth, and increased susceptibility to disease (Oke *et al*., 2024).

Major Causes of oxidative stress in poultry can be listed ad below:

***Ration*:** Unbalanced or insufficient food ration lacking essential antioxidants such as vitamins E and C, or trace minerals such as selenium, can contribute to oxidative stress (Prates, 2023).

***Environmental factors*:** Transfer stress, heat stress, exposure to pollutants, toxins, or even excessive light can lead to increased ROS production (Zhao *et al*., 2018; Oke, 2024; Ayoola *et al*., 2025).

***Infectious diseases*:** Some viral, bacterial, or parasitic infections can trigger oxidative stress by stimulating the bird's immune system (Sorci and Faivre, 2009; Obianwuna *et al*., 2023).

***Compact/intensive production systems*:** High density, insufficient ventilation, or stressful management can cause oxidative stress in poultry special in commercial breeding systems (Gržinić *et al*., 2023).

Antioxidants can be obtained due to variety of supplemnts and feedstuffs with natural and synthetic origin; Natural sources include plant-derived compounds such as vitamins (vitamin E, Vitamin C), carotenoids (beta-carotene, lutein), polyphenols (flavonoids, tannins) and trace minerals (selenium, zinc, copper) (Abbasi *et al*., 2015). In addition, the quality of livestock products such as eggs or meat can also be negatively affected by oxidative stress (Bogolyubova *et al*., 2022). Egg production may decrease, or meat quality may suffer, causing economic losses for the farmer. The solution to overcome the effects of oxidative stress can be done by using stress-reducing drugs. However, the use of drugs in poultry farming for a long time can result in the accumulation of chemicals in the livestock's body, making them dangerous

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if consumed. Antioxidants have a significant effect on the qualitative characteristics of broiler meat. They help stabilize the color of the meat during storage by preventing the oxidation of lipids and maintaining the integrity of muscle tissues (Sohaib *et al.,* 2017). As well as, Antioxidant supplementation can increase tenderness, juiciness and taste of meat, leading to improved consumer acceptance (Ozturk and Dogan, 2017). An alternative that is considered safer is the use of chemical compounds found in plants that have high oxidation activity. One plant that is considered to have the potential to have high antioxidant content is cashew nut shell (*Anacardium occidentale* L.). Cashew nut shell is the main by-product of the cashew nut industry whose processing is still rarely done and has many uses. in medicine and industry. Its documented that cashew nut shell oil has the ability to act as an antioxidant because of the bioactive compounds contained therein, namely anacardic acid, cardol, cardanol, and methyl cardol, which are natural phenolic compounds that can protect blood cells from oxidative damage, by preventing Free radicals can damage cell membranes and can also control the activity of antioxidant enzymes in the blood which help stop oxidation chain reactions (Chang *et al.*, 2016).

MATERIALS AND METHODS

The method used in this research is the extraction and fractionation method for making samples. to test antioxidants and antioxidant enzymes using the DPPH (*1,1-difenil-2-pikrilhidrazil*) method.

***Design and management*:** This research is an experimental study using a Completely Randomized Design (CRD) with 2 treatments with 3 different concentrations for each treatment and 3 replications. The treatment in this study consisted of P1= Cashew nut shell fraction (Concentration 80 ppm, 160 ppm, and 320 ppm) and P2= Control vitamin C (Concentration 80 ppm, 160 ppm, and 320 ppm)**.** Samples of cashew nut shells were cleaned with clean water and then dried in two stages, the first stage under sunlight followed by drying in an oven to reduce the water content contained in the cashew nut shells. After drying, the sample is ground into powder and then extracted using the maceration method. After obtaining the sample yield, it was continued with the fractionation process using ethyl acetate to obtain a sample fraction which was then tested in the laboratory with the parameters of phytochemical fraction test, antioxidant content test and antioxidant enzyme test on the cashew seed shell fraction.

***Determination of phenol levels*:** The total phenol test was carried out using a standard solution of gallic acid in 80% methanol with various concentrations of 0-100 ppm, 100 µL each and 100 µL Follin Ciocalteu reagent was added. Eight hundred microliters of 5% sodium carbonate were added to the standard solution, resulting in a total volume of 1000 microliters, and the mixture was allowed to stand for 90 minutes prior to measurement at a wavelength of 750 nm. The material was extracted using 80% methanol to a final amount of 5 mL, homogenized, and centrifuged at 3000 rpm for 15 minutes to get the supernatant. The supernatant was filtered to get a filtrate. 10 µL of the filtrate was aliquoted, subsequently diluted to a final volume of 100 µL. Then, 100 mL of Folin-Ciocalteu reagent was included, followed by the addition of 800 µL of 5% sodium carbonate, resulting in a total solution volume of 1000 µL. The mixture was allowed to stand for 90 minutes prior to measuring the absorbance. The calculation of total phenol uses the regression equation formula y = ax + b derived from standard measurements. Results are presented as milligrams of gallic acid equivalents per gram of dry weight of extract (mg GAE/g DM extract).

***Determination of flavonoid levels*:** Testing for total flavonoids was carried out using a standard solution of quercetin in 50% methanol with various concentrations of 0-100 ppm of 500 µL each. Five hundred microliters of 2% AlCl3 were incorporated into the standard solution, resulting in a total volume of 1000 microliters, and the mixture was allowed to stand for 90 minutes prior to measurement at a wavelength of 435 nm. The material was extracted using 50% methanol to a volume of 5 mL, homogenized, and centrifuged at 3000 rpm for 15 minutes to get the supernatant. The supernatant was filtered to get a filtrate. 25 µL of the filtrate was extracted and subsequently diluted to a total volume of 500 µL, to which 500 µL of 2% AlCl3 was included, resulting in a final solution volume of 1000 µL. The mixture was allowed to stand for 90 minutes prior to measuring the absorbance. The total flavonoid calculation uses the regression equation y = ax + b derived from the analysis of standard solutions. Results were presented as milligrams of quercetin equivalents per gram of dry weight of extract (mg QE/g DW of extract).

***Antioxidant activity test*:** A total of 2 mL of cashew nut shell ethanol extract with concentrations of 80 ppm, 160 ppm, and 320 ppm was added to 2 mL of DPPH solution in ethanol each and vortexed for 5 seconds. The change from purple to yellow indicates the efficiency of fighting free radicals. The absorbance was measured on a UV-Vis spectrophotometer with a wavelength of 517 nm, after incubation for 30 minutes. Then observe the comparison with vitamin C as a standard.

***Determination of IC50 of cashew fruit seed shell extract*:** In a measuring flask with a capacity of 5.0 milliliters, a number of stock solutions containing cashew nut shell extract and vitamin C were deposited. These stock solutions were of three different concentration series: 80 milliparts per million, 160 parts per million, and 320 parts per million. Following this, 0.7 mL of 0.4 mM DPPH was added to the sample, then ethanol was applied to the mark using the same method. Following a thirty-second period of vertexing, the mixture was then incubated for thirty minutes. I measured the absorbance of the sample by comparing it to a blank that was composed of a stock solution in ethanol at the maximum

absorbance (λmax). In addition, when compared with Vitamin C controls with different concentration series, namely 80 ppm, 160 ppm, and 320 ppm, with 0.7 mL of 0.4 mM DPPH in ethanol p.a. up to the mark, the results in this study were significantly different. After that, the percentage of antiradical activity was determined. In order to get a linear regression equation that can be used to predict the sample concentration at 50% activity, it is necessary to create a linear regression curve that maps concentration against the percentage of antiradical activity. The test for antiradical activity was carried out three times and reproduced. In addition, the stock preparation and sample dilution processes were repeated three times as part of each test.

***Antioxidant enzyme activity test*:** Analysis of antioxidant enzyme activity in extracts can be carried out using quantitative methods that reflect the capacity of enzymes to destroy or reduce antioxidant compounds.

a. Superoxidase Dismutase (SOD) Enzyme Activity Test

Analysis of peroxidase enzyme activity: 0.3 ml of supernatant was taken and added to a solution containing 2.1 ml of 0.1 M PO4 buffer pH 6.8; 0.3 ml guaiacol 1.6% and 0.3 ml H2O2 0.04 M. The activity of peroxidase was determined by measuring the exhaustion of H2O2 within one minute using a spectrophotometer with a wavelength of 470 nm.

b. Catalase Enzyme Activity Test

Analysis of catalase enzyme activity by adding 0.04 ml of supernatant to a solution containing 2.56 ml, 50 Mm Potassium Phosphate Buffer pH 7 and 0.4 ml 15 Mm H2O2 within one minute using a spectrophotometer with a wavelength of 240 nm.

c. Glutathione Peroxidase (GPx) Enzyme Activity Test

A total of 20 µl of extract supernatant was added with 200 µl of 0.1 M phosphate buffer pH 7 containing 0.1 mMEDTA, 200 µl of 10 mM reduced glutathione (GSH) and 200 µl of glutathione reductase enzyme (2.4 units). Incubate for 10 minutes at 370C then add 200 µl of 1.5 mM NADPH and incubate again for 3 minutes at the same temperature and continue with the addition of 200 µl of H2O21.5 mM. The rate of change in absorption during the conversion of NADPH to NADP+ was recorded spectrophotometrically at a wavelength of 340 nm for 3 minutes. GSH-Px activity is expressed as µmol NADPH oxidized to NADP+min-1mg-1protein with an extrinsic coefficient of 6.22mM-1cm-1.

RESULTS AND DISCUSSION

***Organoleptic test of extract*:** The organoleptic test aims to see the physical appearance of a preparation which includes shape, color and odor as shown in Table 1.

The results of the organoleptic test on the cashew nut shell fraction showed that it was thick, blackish brown in color according to the color of the cashew nut shell that had been dried and lost its water content, and the resulting odor was distinctive. The aroma or odor and color produced by the cashew nut shell fraction is influenced by the concentration of the extract used. According to Juwita *et al.* (2013) the higher the concentration of the extract, the aroma or distinctive odor increases and the color of the extract becomes more intense. The National Standardization Agency (1996) determined the organoleptic standardization of herbal extracts to show a dark color, thick texture, characteristic odor of herbal ingredients, and soluble in water.

Table 1. Organoleptic test of cashew seed shell fractionation.

Organoleptic test of extract	Keterangan
form	Thick
Color	Dark Chocolate
Smell	distinctive smell

***Test for phenol content*:** The total phenol content test aims to determine the amount of phenol contained in the sample. The total phenol content in the cashew nut shell fraction is 160 mg/g Galak acid. The total phenol content was obtained from the equation y = 0.0011x+0.2624 (Fig. 1) which shows an increase at each concentration. Antioxidant activity is directly proportional to total phenol, the higher the phenol content in a material, the higher its activity as an antioxidant (Huang *et al.*, 2015). This is also supported by Hadriyanto's (2011) research on mangosteen peel (*Garcinia mangostana* L) which shows that there is a directly proportional relationship between total phenol content and antioxidant activity.

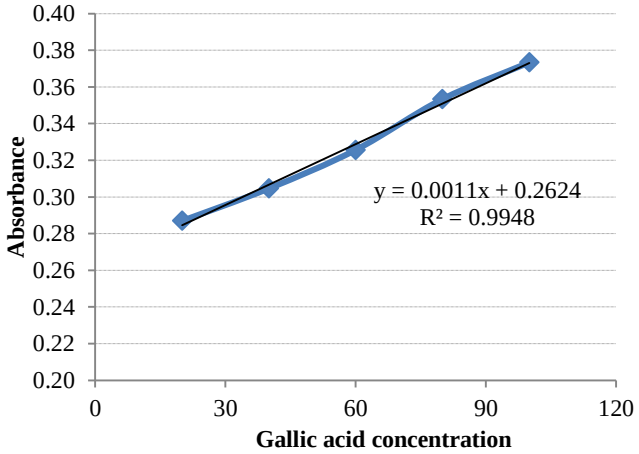

Figure 1. Graph of the relationship between gallic acid concentration and absorbance.

In general, compounds that have bioactivity as antioxidants are phenol group compounds. Phenolic compounds inhibit free radicals by donating protons and forming stable radicals. The formation of this stable radical is due to the free electrons in the radical being stabilized by electron delocalization due to resonance in the aromatic ring. Apart from that, based on phytochemical tests, it is known that the ethyl acetate fraction

contains phenolic compounds, flavonoids, alkaloids and saponins (Yuliawan *et al.*, 2021). The presence of these compounds causes the ethyl acetate fraction from the skin of cashew nuts to act as an antioxidant.

***Flavonoid content test*:** The total flavonoid content found in the cashew nut shell fraction is 300 mg/g quarcetin. The total phenol content is obtained from the equation y = 0.0011x + 0.4001 (Fig. 2) which shows an increase at each concentration.

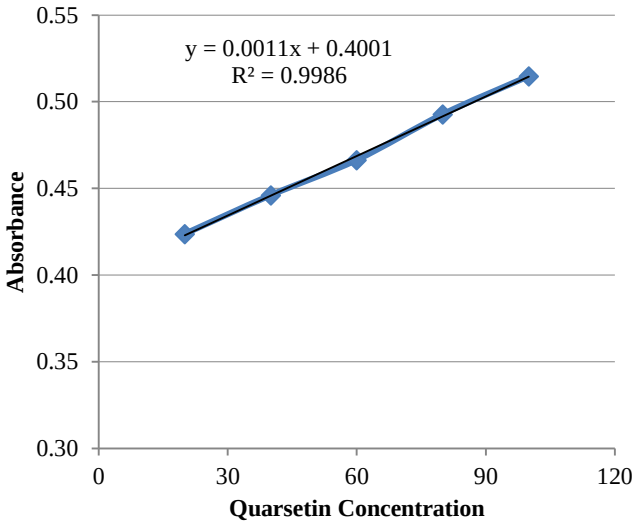

Figure 2. Graph of the relationship between quercetin concentration and absorbance.

The ability of the cashew nut shell fraction to reduce an antioxidant is expressed by the percent value of reduction capacity. The higher the reduction percentage indicates that the compound has potential as an antioxidant. Antioxidants derived from flavonoid compounds will react with the fractions through an oxidation-reduction mechanism. Flavonoid compounds will experience oxidation while cerium will experience reduction (Rahayu *et al.*, 2015).

***Antioxidant content test*:** The results of the phytochemical screening test showed that the cashew nut shell fraction contained flavonoid and phenolic chemical compounds. Where the chemical compounds flavonoids and phenolics are one of the chemical compounds that have the potential to act as antioxidants, based on these results it shows that the cashew nut shell fraction has the potential to act as an antioxidant. The antioxidant activity of the sample resulted in a change in the color of the DPPH solution in ethanol from dark purple to pale yellow. The parameter used to indicate antioxidant activity is Inhibitory Concentration (IC50), namely the concentration of an antioxidant substance that provides an inhibition percentage of 50%. The smaller the IC50 value means the stronger the antioxidant activity, the level of antioxidant strength of the test compound using the DPPH method can be classified based on the IC50 value, very strong with IC50 <50 ppm, strong IC50 50-100 ppm, medium IC50 101-150 ppm, and weak IC50 > 150 ppm (Molyneux, 2004).

Table 2. Inhibition % value and IC50 value of cashew nut shell fraction.

Sample	Conc.	Absorbance	% Inhibition	IC50 value (ppm)
Extract	80 ppm	0.336	48.571	99.459
	160 ppm	0.304	53.469	
	320 ppm	0.275	57.959	
Control (vit. C)	80 ppm	0.319	51.224	37.561
	160 ppm	0.288	55.969	
	320 ppm	0.252	61.480	

The results obtained by the concentration value of the cashew nut shell fraction are directly proportional to the inhibition value. The higher the concentration, the higher the inhibition value. This also shows that the greater the concentration, the more antioxidant content in the extract which can reduce free radical activity, indicated by the decay of the purple color of DPPH (Widyasanti *et al.*, 2016). The results show that inhibition and extract concentration have a high correlation at a concentration of 320 ppm of 57.959%. Antioxidant testing was also carried out on vitamin C (ascorbic acid) as a positive control and comparison. Control is intended to test the validity of a method, comparing research results with another research that has been conducted (Widyasanti *et al.*, 2016). The correlation between concentration and inhibition of vitamin C is depicted in Figure 7 and has a high correlation of 51.22-61.480% at each concentration. If the antioxidant compound content is greater, the ability to inhibit free radicals will be higher (Putri and Khonsa, 2022).

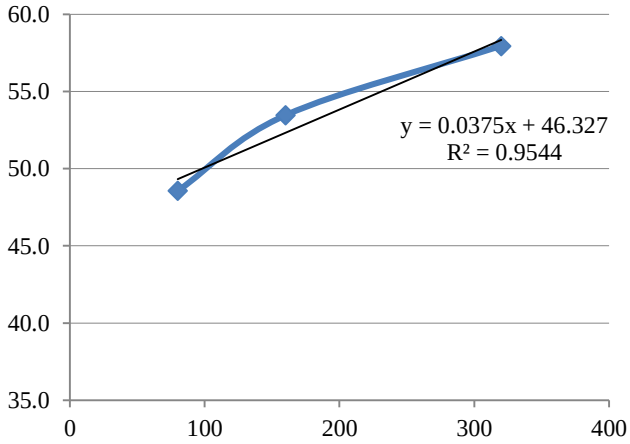

Figure 3. Standard curve of antioxidant content of cashew nut shell fraction.

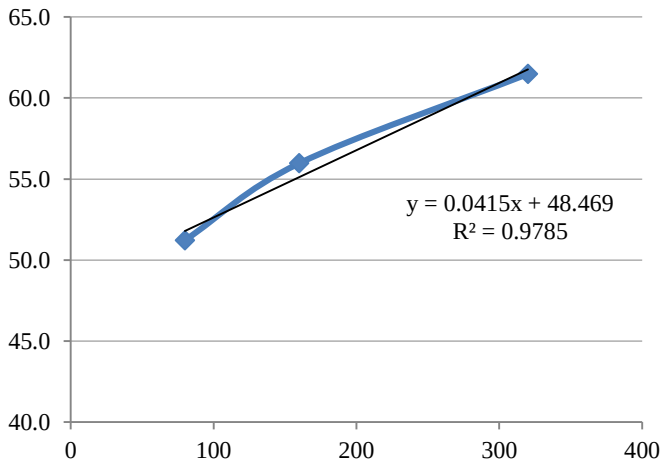

Figure 4. Standard curve for vitamin C antioxidant content.

According to Molyneux (2004), compounds are considered to have extremely high antioxidant activity if the IC50 value is less than 50 ppm. The strong IC50 group is comprised of compounds with an IC50 value between 50 and 100 ppm, the medium group for compounds with an IC50 value between 101 and 150 ppm, and the weak group for compounds with an IC50 value between 150 and 200 ppm. On the basis of this statement, it is possible to assert that the cashew nut shell fraction possesses a high antioxidant content, as shown by an IC50 value of 99,459 ppm, and that vitamin C possesses a very high antioxidant activity, as indicated by an IC50 value of 37,561 ppm.

Antioxidant enzyme test

***SOD enzyme test*:** Cashew nut shells contain bioactive compounds such as phenols and flavonoids found in the extract which work synergistically with the activity of the SOD enzyme, providing a protective effect against oxidative stress. The SOD enzyme acts as a natural antioxidant that protects cells from damage caused by free radicals, especially superoxide radicals (Amico *et al.*, 2022).

Table 3. Superoxide Dismutase (SOD) enzyme test for cashew nut shell fractions.

Sample	Conc.	Absorbance	% Inhibition	IC50 value (ppm)
SOD	80 ppm	0.224	42.134	
Enzyme	160 ppm	0.217	48.617	206.250
	320 ppm	0.185	55.968	

In testing the cashew nut shell fraction, it was seen that the absorbance value decreased with the addition of the concentration value in the sample. This decrease in absorbance value indicates a reduction in the concentration of free radicals due to inhibition by the sample. The lower the absorbance value, the higher the percentage of inhibition that occurs in the sample (Hidayati and Maskuroh, 2023). The linear regression equation can be seen from the cashew nut shell fraction curve in Fig. 5.

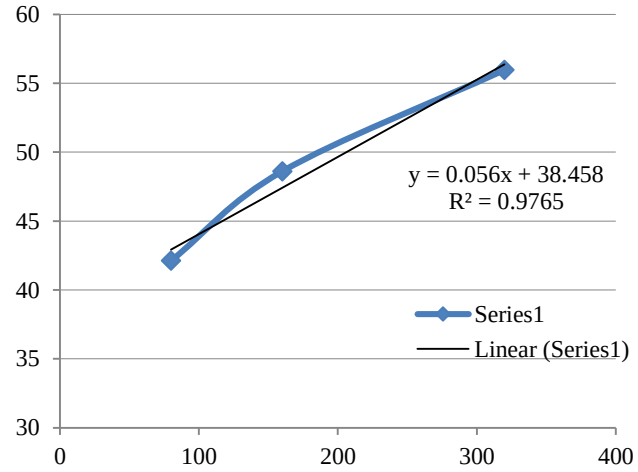

Figure 5. Standard curve of cashew nut shell fraction.

Based on the table, you can see the IC50 value for the concentration of an antioxidant substance, namely the one that provides 50% inhibition or reduction of free radicals. The results of testing antioxidant enzyme activity in the cashew nut shell fraction showed an average IC50 value of 206.250 ppm. Based on the level of antioxidant strength of the test compounds using the DPPH method, they can be classified based on if the IC50 value is very strong with IC50 <50 ppm, strong IC50 50-100 ppm, medium IC50 101-150 ppm, and weak IC50 >150 ppm (Hidayati *et al.*, 2023).

***Catalase Enzyme test*:** Catalase is an enzyme that functions to break down hydrogen peroxide (H_2O_2), a reactive compound that can cause damage to cells if it is not immediately broken down into water and oxygen. This catalase activity is very important in protecting cells from the negative effects of free radicals and oxidative stress, which are often associated with degenerative diseases in livestock. Enzymatic antioxidants found in the body, including catalase, act as the body's first line of defense against reactive oxygen species. This is in accordance with research by Untari *et al.* (2014) which states that the catalase enzyme will catalyze the decomposition of one of the ROS, namely hydrogen peroxide, into water and oxygen so that it can protect cells from oxidative damage.

Table 4. Catalase enzyme test of cashew nut shell fraction.

Sample	Conc.	Absorbance	% Inhibition	IC50 value (ppm)
Katalase	80 ppm	0.126	46.393	
Enzyme	160 ppm	0.109	53.890	121.493
	320 ppm	0.087	62.942	

The IC50 results in testing the antioxidant enzyme (catalase enzyme) in the cashew nut shell fraction which had been replicated 3 times resulted in a result of 121.493 ppm. Based on research results, the cashew nut shell fraction has antioxidant activity in the medium category. This indicates that cashew nut shells have potential as a source of natural antioxidants that can protect body cells from oxidative stress. Based on the level of antioxidant strength of the test compounds using the DPPH method, they can be classified based on if the IC50 value is very strong with IC50 <50 ppm, strong IC50 50-100 ppm, medium IC50 101-150 ppm, and weak IC50 >150 ppm (Hidayati *et al.*, 2023).

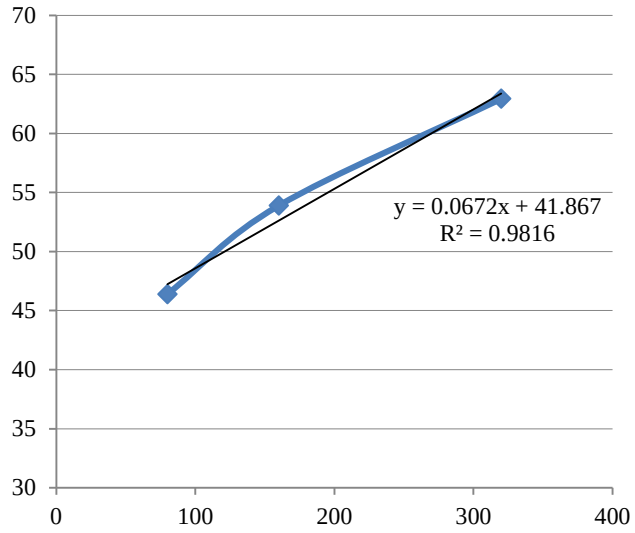

Figure 6. Standard curve of the catalase enzyme for the cashew nut shell fraction.

***GPx enzyme test*:** Phenolic compounds contained in the cashew nut shell fraction, such as anacardic acid, have strong antioxidant properties. This compound can stimulate the body to increase the production of antioxidant enzymes such as GPx, superoxide dismutase (SOD), and catalase (CAT). Thus, consumption of cashew nut shell extract or fraction through feed, for example in chickens or even in the context of human health, can increase the activity of endogenous antioxidant enzymes, including GPx (Margarete, 2020).

Table 5. Glutathione peroxidase (GPx) enzyme test of cashew nut shell fraction.

Sample	Conc.	Absorbance	% Inhibition	IC50 value (ppm)
Enzim	80 ppm	0.224	34.913	
GPx	160 ppm	0.194	43.617	279.143
	320 ppm	0.163	52.515	

Compounds are said to have very strong antioxidant activity if the IC50 value is less than 50 ppm, the strong IC50 group is between 50-100 ppm, the medium group if the IC50 value is 101-150 ppm, and the weak group if the IC50 value is between 150-200 ppm (Molyneux, 2004).

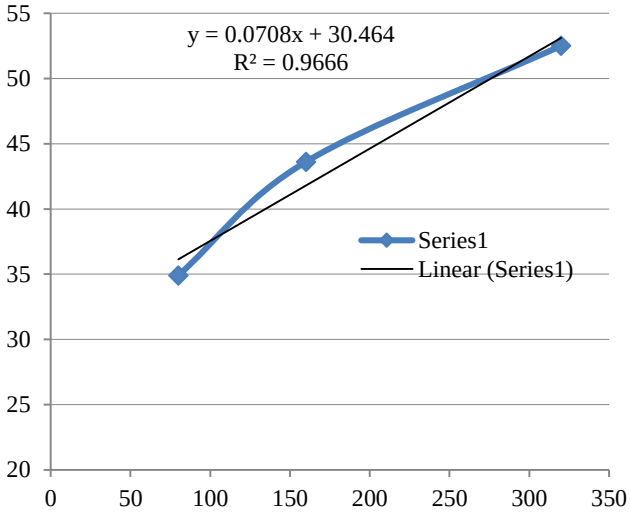

Figure 7. Enzyme standard curve (GPx) of cashew nut shell fraction.

***Conclusion*:** Taken together, it can be concluded that the cashew nut shell fraction can potentially be used as an antioxidant product in poultry feed. The research results showed that certain cashew nut shell fractions from cashew nut shells contained bioactive compounds that were able to increase GPx activity in the weak category with an IC50 value of 279.143 ppm. In a material, its higher activity as an antioxidant is in accordance with the results of the antioxidant test of the cashew nut shell fraction which has an antioxidant content at a strong level with an IC50 value of 99.459. Using cashew nut shell fractions is suggested for elimination of oxidative stress in poultry.

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***Authors' contribution*:** All of authors were actively pariticipate in conducting, drafting, and writing of manuscript.

***Conflict of interest*:** All authors declare no conflict of interest.

***Availability of data*:** Raw data can be available when requested by journal.

***SDGs addressed*:** Good Health and Well-being, Responsible Consumption and Production

***Policy referred*:** National-Level Policies, National Research Master Plan (RIRN).

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